2011 2nd International Conference on Agricultural and Animal Science
IPCBEE vol.22 (2011) © (2011) IACSIT Press, Singapore

Transformation of *Agrobacterium Tumifascience* LBA 4404 with a Cholin Oxidase-Cox Gene Conferring Salinity Tolerance

Ashutosh Vadawale, Ritu Mihani, Asha Mathew and Pushpa Robin

Department of Biochemistry, Faculty of Science,
The Maharaja Sayajirao University of Baroda, Vadodara - 390 002. India

Abstract. Agrobacteium tumifascience strain LBA 4404 was transformed with the cholin oxidase gene through triparental mating. The growth of transformants was checked under appropriate antibiotic selection pressure. The successful tranformants were confirmed by the ketolactose test. The phenotypic expression of *cox* gene was checked by growing *Agrobacterium* and *Agrobacterium* transformants on medium containing different concentration of NaCl. The transformants were able to survive in presence of 300mM NaCl while non-transformants (control) could only grow and survive up to a NaCl concentration of 100 mM indicating that the tolerance capacity of the transformants was increased by 200mM as compared to control.

Keywords: Salinity, Cholin Oxidase, transformation

1. Introduction

Glycine betaine (GB) is one of the most potent compatible solutes which protect the cell machinery in plants against various kinds of stresses like salinity, drought, cold etc. (Hayashi et al 1998). Among the plants which can naturally produce GB, three pathways exist for its synthesis (Hayashi et al 1998). It starts with choline and proceeds through a reaction that involves one or two enzymes for the oxidation of choline to GB. Different enzymes catalyze the reaction in different organisms. The overall reaction is

Choline ----------→ Betaine aldehyde ----------→ GB
CMO / COX / CDH *COX / BADH*

CMO : Choline monooxygenase, COX :Choline oxidase, CDH Choline dehydrogenase, BADH : Betaine aldehyde dehydrogenase.

COX pathway has the advantage of being a single enzyme which can catalyze both steps and also it does not require any cofactor for catalysis (Sakamoto et al, 2001).

2. Materials and Methods

The gene *cox* (choline oxidase) gene was received as kind gift from Dr. Gopalan Selvraj, Plant Biotechnology institute, NRCC, Canada in the form of Binary vector pHS724 and was amplified in *E. coli* DH5α through $CaCl_2$ method. In order to transform this gene of interest into the Agrobacterium tumefaciensce, triparental mating was carried out between *E. coli* DH5α bearing gene of interest (doner strain), *E. coli* DH5α bearing plasmid pRK2013 (helper strain) and the Agrobacterium LBA4404.

3. Culture Maintenance

E. coli DH5α strains which was used for transformation of ligation mixtures and maintaining recombinant plasmids were maintained on Luria agar plates. *E. coli* DH5α bearing helper plasmid pRK2013 which was used as a helper strain for mobilizing a donor plasmid contained in another *E. coli* DH5α strain was maintained on Luria agar plates containing 50 μg/ml kanamycin as the plasmid pRK2013 has a kanamycin resistance gene. E.coli DH5α clones harbouring pHS724 recombinant plasmid were maintained on LA plates with 50 μg/ml kanamycin. *Agrobacterium tumefaciens* LBA4404 was maintained on AB

Medium containing 5 µg/ml rifampicin & 10 µg/ml tetracycline as the strain LBA 4404 is resistant to rifampicin & tetracycline antibiotics.

4. Plasmid Extraction

Plasmid extraction was done by Alkaline Lysis method of Sambrook *et al.* (1989) (Miniprep). A single colony of transformed bacteria was inoculated in 5 ml of LB with antibiotic. The culture was incubated overnight at 37°C under vigorous shaking condition on shaker for a 24 hrs. Next day the culture was centrifuged at 12000 rpm for 10 min. The supernatant was discarded & 100 µl of ice cold ALS-I was added to resuspend the pellet by vortexing. 150 µl ALS-II was added & the contents of the tube were mixed by inverting 5 times, the tube was stored on ice for 10min. 200 µl ALS-III was added & mixed by inverting the tubes 5 times, tube was stored on ice for 15-20 min. Centrifugation done at 12000 rpm for 10 min, the supernatant was transferred to a fresh microfuge tube and equal volume of Isopropylalcohol was added. The tube was incubated at -20^{o}C overnight. Centrifugation was done at maximum rpm for 10min.The supernatant was carefully discarded & 200 µl 70% alcohol was added & inverted gently 2 times & centrifuged for 2 min, then the alcohol was removed carefully. The microfuge tube was kept in an inverted position to completely remove last traces of alcohol. 20 µl Triple distilled water was added and kept at room temperature for 15 min for DNA to dissolve. The purity of the plasmid preparation was checked by agarose gel electrophoresis

5. Competent Cell Preparation

The protocol from Sambrook *et al*. (1989) was used for preparation of competent E. coli with an efficiency of ~10^6 transformed colonies/ µg of supercoiled plasmid DNA. A single bacterial colony was inoculated in 5 ml of LB and the culture was incubated overnight at 37°C with vigorous shaking. 1 ml of overnight grown culture was transferred in 100 ml LB and incubated at 37°C with vigorous shaking till the OD_{600} reaches 0.4. The bacterial cells were transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The cultures were cooled to 0°C for 10 min. Cells were recovered by centrifuging at 4500rpm for 10 min at 4°C. The medium was decanted from cell pellet & tubes were placed in an inverted position for 1 min to allow last traces of the media to drain away. Pellet was re-suspended by swirling or gentle vortexing in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution. Cells were recovered by centrifuging at 4500rpm for 10min at 4°C. Medium was decanted from cell pellet; tubes were placed in an inverted position for 1 min to allow last traces of the media to drain away. Pellet from each tube was re-suspended by swirling or gentle vortexing in 1ml of 0.1 M $CaCl_2$ and 1ml of 40% glycerol. The cells were directly used for transformation.

6. Transformation Using $CaCl_2$

1*0*0-200 µl $CaCl_2$ treated cells were transferred to a sterile chilled polypropylene tube. Upto 5 µL of DNA sample or ligation system was added to the tube and the contents mixed by swirling gently. The tube was stored on ice for 30 min. The tube was transferred to a rack and kept in preheated 42°C water bath and incubated exactly for 90 sec without shaking. The tube was rapidly transferred to an ice bath to chill for 2 min. About 400-800 µl of LB medium was added accordingly to the tube and incubated for 45 min at 37°C. Appropriate volume of cells was plated onto pre-warmed LB plates with appropriate antibiotic. The plates were incubated at 37 °C for 16-18 hours.

7. Triparental Mating

Triparental mating was carried out using *E.coli* DH5α with pHS724 (kan^R) as the donor strain, E.coli DH5α with pRK2013 (kan^R) as the helper strain and Agrobacterium LBA4404 (Rif^R,Tet^R) as the recipient as per the procedure described by Hoekema et al. 1983.Four days prior to the triparental mating, A *tumefaciens* was streaked to obtain a single colony on Luria agar plates which contained rifampicin & tetracycline and was incubated at 30 °C. *E. coli* DH5α harboring pRK2013 & *E. coli* DH5α harboring the plasmid to be mobilized were streaked before one day to obtain a single colony on LB agar with 50µg/ml of Kanamycin. On the day of the triparental mating, a plate of Luria agar without any antibiotic was prepared. One colony each from *E. coli* DH5α bearing pRK2013, *E. coli* DH5α harboring the plasmid which has to be mobilized and *A. tumefaciens* was patched separately on Luria agar plate very close to each other. With a sterile loop,

all the three bacterial strains were mixed very well and the plate was left at 30 °C for 12-18 hrs. On the second day from the procedure, six culture tubes which contained 0.9ml LB were autoclaved and kept ready. Six plates containing Luria agar plate with relevant antibiotics were poured and kept ready. After mating, the bacteria on the Luria agar plate were scrapped and suspended in 1ml LB. A serial dilution was performed by transferring 0.1ml of bacterial suspension into 0.9ml of LB. Likewise 4-5 dilutions were made up to 10^{-4}/ 10^{-5}. 100 µl of each dilution was added to Luria agar medium with appropriate antibiotics and was spread uniformly. The plates were incubated for 4-5days.

8. Results and Discussion

8.1. Transformation of pHS724 into *E.coli* DH5α

$CaCl_2$ mediated transformation was carried out in order to amplify the plasmid using the vector E.coli DH5α. The presence of the plasmid in E.coli DH5α was checked by restriction digestion pattern as well as phenotypic expression (kanamycin resistance).

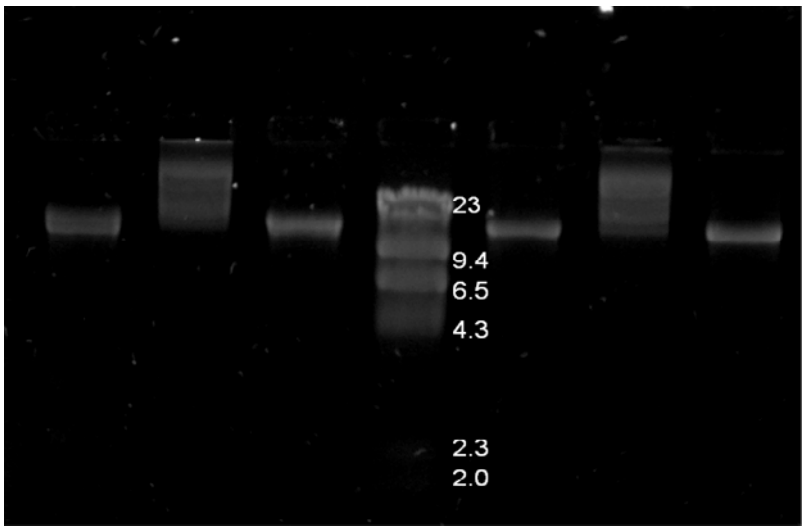

Fig. 1 : Confirmation of the plasmid by agarose gel electrophoresis.

Lane1:0.2µl Hind III digest Lane 2: Undigested plasmid
Lane 3:0.4µl Hind III digest Lane 4: λ Hind III marker
Lane 5:0.2µl Eco RI digest Lane 6: Undigested plasmid
Lane 7:0.4µl Eco RI digest

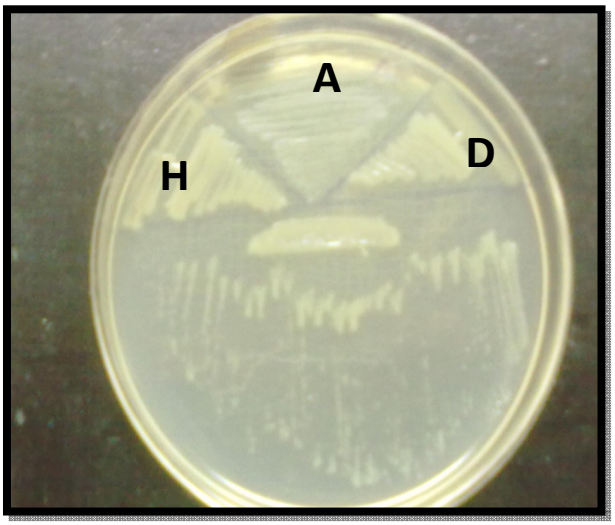

Fig. 2: Triparental mating (A: Agrobacterium tumefaciens (Rif^R Tet^R); D: Donor pHS724 (Kan^R)H: Helper strain (Kan^R)

8.2. Transformation of pHS724 into *Agrobacterium tumefaciens* LBA4404

Table 1: Resistance of plasmids to antibiotics

	Kan	Rif-Tet	Rif-Tet-Kan
pHS724	√	-	-
pRK2013	√	-	-
Agrobacterium	-	√	√
Agrobacterium transformants	√	√	√

After two days of incubation, sufficient growth was obtained. All the three cultures (also streaked individually) showed adequate growth. Growth obtained was subjected to selection pressure by spreading different dilutions of antibiotics rifampicin, tetracycline and kanamycin on Luria agar. *Agrobacterium tumefaciens* transformants survived because kanamycin present in the medium did not allow the growth of non-conjugated *Agrobacterium tumefaciens* cells. *Agrobacterium tumefaciens* LBA4404 is sensitive to kanamycin but resistant to rifampicin and tetracycline. Rifampicin and tetracycline did not allow the growth of both *E.coli* DH5α pHS724 transformants & *E.coli* helper strain as they are sensitive to rifampicin and tetracycline though resistant to kanamycin.

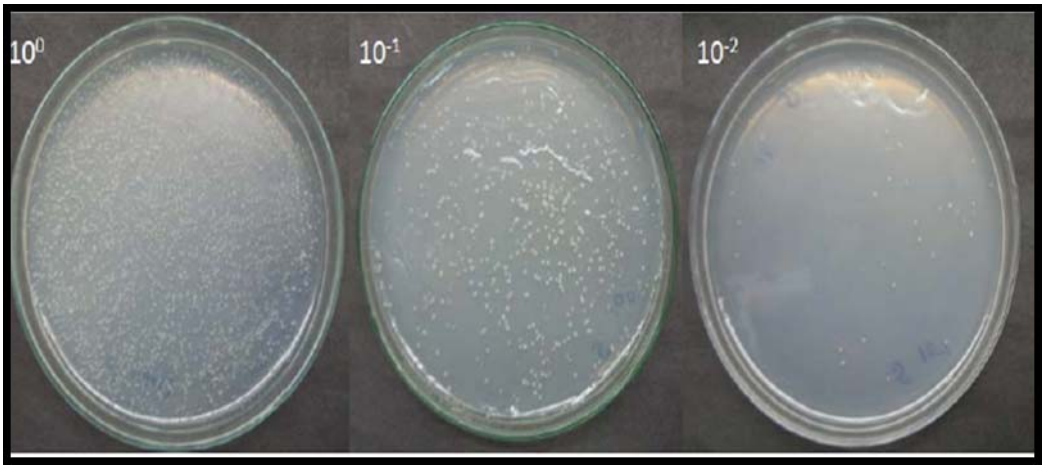

Fig. 3: *Agrobacterium tumefaciens* on LA plate

Table 2: Growth of transformants on Luria Agar plate

DILUTION	NO. OF COLONIES	cfu/ml
Undiluted	**750**	7.5×10^2
10^{-1}	250	2.5×10^2
10^{-2}	20	2×10^2

Agrobacterium and *Agrobacterium* transformants were streaked on the same LA plate containing antibiotics rifampicin, tetracycline and kanamycin. *Agrobacterium* was unable to grow as it was sensitive to kanamycin while sufficient growth of *Agrobacterium* transformants was obtained. Growth obtained could be that of *A. tumefaciens* cells which had received the binary vector construct. These could be putative pHS724 *A.tumefaciens* transformants. The *A. tumefaciens (*strain LBA 4404) transformation with the construct (pROK-ITCP17) has also been reported by triparental mating using a helper plasmid pRK 2013 by Raj et al. (2005).

8.3. Confirmation of *A.tumefaciens* transformants by Ketolactose test

The *A. tumefaciens*, ketolactose colony was confirmed through **Ketolactose/** Benedict's test (Bernaerts et al. 1963) which is a confirmatory test given positive only by *A. tumefaciens* but negative by *E.coli*. Therefore, a positive Benedict's test confirms the culture as *A. tumefaciens*. A culture giving positive ketolactose test showed a yellow ring around its colony when flooded with Benedict's reagent due to the formation of ketolactose which reacts with Benedict's reagent to form yellow colour. As can be seen in the fig 4, a yellow colour was observed around the colony which confirmed that the culture was *A. tumefaciens*.

Fig 4: Ketolactose test

Confirmation of the transformation of plasmid pHS724 into *Agrobacterium* was done by checking the phenotypic expression of *cox* gene in presence of NaCl.

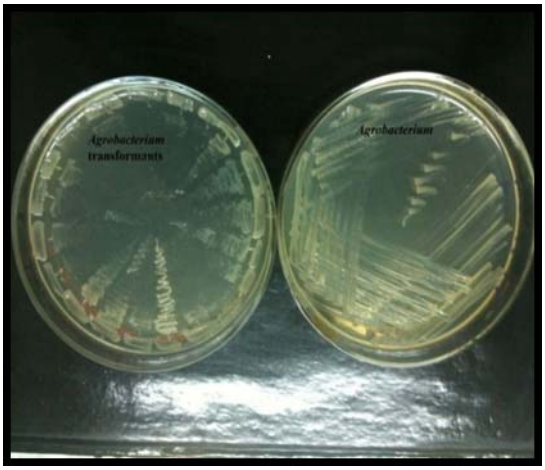

Fig 5a

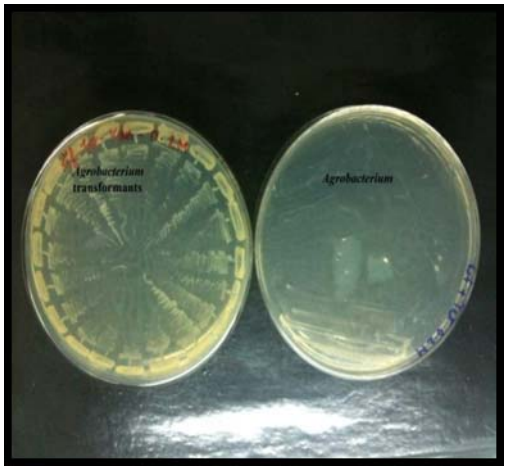

Fig 5b

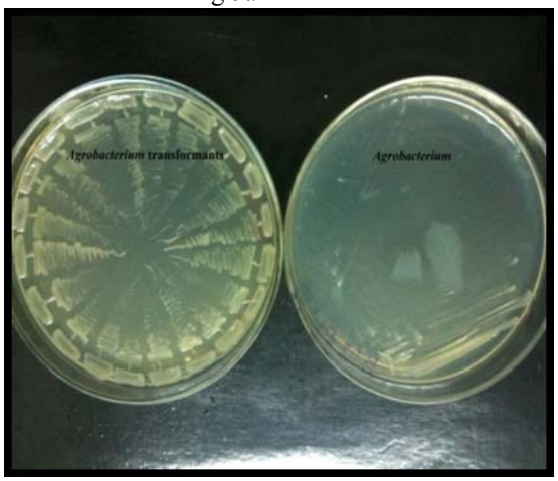

Fig 5c

Fig 5d

Fig. 5. *Agrobacterium* and *Agrobacterium* transformants on a medium containing

a: 0.1M NaCl, b: 0.2M NaCl, c: 0.3M NaCl. d: 0.4M NaCl.

8.4. Expression of Choline Oxidase (*cox*) in *Agrobacterium*

Expression of *cox* gene in *Agrobacterium* was checked by streaking the culture of Agrobacterium transformants containing pHS724 on a medium containing NaCl along with the antibiotics rifampicin, tetracycline and kanamycin. Also, the growth of Agrobacterium on a medium containing NaCl and antibiotics rifampicin and tetracycline was checked

9. Discussion

An attempt was made to incorporate the choline oxidase gene responsible for production of glycine betaine, an osmoprotectant into *Agrobacterium tumefascience*- LBA4404. The plasmid containing the gene (pHS724) was amplified in the vector *E.coli* DH5α. Primary selection of the transformants was done by the presence of kanamycin resistance. The transformants were confirmed by plasmid isolation and further by restriction digestion pattern.

However, the expression of *cox* gene in the vector *E.coli* DH5α could not be observed. This is due to the absence of inducible promoter which can be expressed in the bacteria *E.coli*. Since the gene choline oxidase is under the control of viral promoter 35S, no difference in the salt tolerance capacity was observed in *E.coli* DH5α transformants as compared to *E.coli* DH5α (control).

Further triparental mating of *E.coli* DH5α containing the plasmid pHS724 (serving as the donor strain) was performed with *Agrobacterium tumefaciens*; while *E.coli* DH5α containing the plasmid pRK2013 served as the helper strain thereby mobilizing our gene of interest to *Agrobacterium tumefaciens*.

The transformants were confirmed by their ability to survive in presence of all the three antibiotics kanamycin, rifampicin and tetracycline. Phenotypic expression of the transformants was done by streaking the transformants in presence of NaCl. The transformants were able to survive in presence of 300mM NaCl while 400mM showed no growth. Hence the salinity tolerance capacity of the transformants was increased by 200mM as compared to control. Our results matches with Deshnium et al.(1995) who also observed increased salinity tolerance up to 400mM NaCl in Synechococus when transformed with *codA* gene for cholin oxidase. Increased salinity tolerance up to 150 and 300mM NaCl has also been reported by Huang et al. (2000) in *Nicotiana tabacum* and *Brassica napus* when transformed with the choline oxidase gene.

In conclusion, the *Agrobacterium tumefaciens* transformants containing the cholin oxidase gene were successfully generated which can be utilized for further transformation in to variety of crop plants of economic importance for conferring salinity tolerance.

10. Acknowledgements

Authors are thankful to Dr. Gopalan Selvraj, Plant Biotechnology institute, NRCC, Canada for providing the cNDA clone of *cox* (choline oxidase) gene and to the GSFC Science Foundation for funding the project.

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